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THE EFFECTS OF PRENATAL MATERNAL STRESS ON PIGLET BIRTH WEIGHT, HEALTH & IMMUNE FUNCTION

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The effects of prenatal dexamethasone administration; a model for prenatal maternal stress, on piglet performance, health and immune function

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SUMMARY

Experimental Design:

The primary objective of this experiment was to investigate the impact of prenatal maternal stress in early- and mid-pregnancy sows on post-natal immune function, performance and health of their offspring. The secondary objective was to assess the impact of birth weight on post-natal health and immunologic function. Prenatal stress was artificially induced in pregnant sows using dexamethasone (Dex) at 0.2 mg/kg intramuscularly (IM) on gestation days 30-36 (Dex-30; n = 27) or 60-66 (Dex-60; n=27). Treated and non-treated control (CTRL; n=25) sows were induced to farrow on day 115. Production parameters including litter size, weight and body confirmation at birth, growth rate, morbidity and mortality rate were compared between Dex-treated and CTRL litters or piglets where appropriate. Organ weights, the stress response to natural weaning, and numerous immunological parameters including lipopolysaccharide-induced inflammatory cytokine production, passively acquired maternal antibody level, leukocyte subtypes, and active immune responses were evaluated in a subset of progeny originating from each Dex-treated and CTRL litter.

Results:

Salivary dexamethasone ELISA levels measured prior to and during the period of dexamethasone treatment were similar across all treatment groups, and dexamethasone treatment had no adverse impact on litter size. Compared to the CTRL group, piglets born in the Dex-30 group were of long-thin body conformation, and had a significantly lower birth weight:crown-rump length ratio (BW:CRL) and body mass index (BMI). The adrenal:brain weight ratio was significantly lower in the Dex-60 compared to the Dex-30 group, and trended lower compared to CTRL pigs, suggesting prenatal dexamethasone had a negative impact on adrenal gland development. There were no group differences in baseline cortisol level, or in the cortisol

response following weaning. The absolute number of $CD8^+$ lymphocytes was significantly lower in the Dex-60 compared to CRTL piglets suggesting pre-natal stress impacted the development or maturation of this vital subpopulation of immune cells. Maternal dexamethasone administration between gestation day 60-66 resulted in faster growth rates during the nursery period from days 20-50 of age. Birth weight (BW) also positively impacted growth rates but from birth to 50 days of age. Moreover, the risk of treatments during lactation was higher, and passively acquired maternal antibody levels lower in low compared to high BW piglets. Compared to high BW piglets, low and average BW piglets had significantly smaller circulating populations of $CD3^+$, $CD8^+$ and gamma-delta lymphocytes, and red blood cell counts at 13 days of age. $CD4^+$ and $CD21^+$ lymphocytes were also significantly lower in low versus high BW piglets. Low and average BW piglets had lower red blood cell (RBC) counts and the RBC's were larger and more variable in size, suggesting the premature release of immature red blood cells into the general circulation.

Conclusions:

Pre-natal dexamethasone treatment appears to alter fetal development and may impact post natal growth. However, we found minimal evidence that it negatively impacted the function of the post-natal immune system or stress response. By contrast, the results of this experiment indicate widespread potentially adverse changes in the immunological and haematological systems of young piglets associated with below average birth weight and altered fetal development.

INTRODUCTION

The Saskatchewan swine industry has expanded rapidly in the last decade, and is a prominent contributor to the value-added agribusiness sector in the province. Competitive feed prices, abundant land mass, and superior reproductive productivity as led to the development large farms often using health technologies such as three-site production and segregated weaning. However, the expansion has coincided with the public's heightened awareness of public health and animal welfare issues including the waning acceptance of stalled gestation housing. Moreover, disease control has become more complex in large farms due to the use of larger rooms, the mixing of pigs from different sources, and the negative impacts of stress on the immune response and disease resistance.

Prenatal programming, a novel area of animal research, is intimately linked to all of the above, and potentially explains some of the phenotypic variation in health or performance that occurs in animal populations. Prenatal programming is a process in which an adverse event such as maternal stress or placental inefficiency occurs at a critical period of fetal development and results in fetal growth retardation, and adverse lifelong changes in the performance and health of the offspring. It is common to all mammalian species, and in humans implicates the fetal origin of adult-onset diseases including hypertension, obesity, insulin resistance and type 2 diabetes, and some reproductive disorders.[1-3] In swine, prenatal programming associated with low birth weight or long-thin body confirmation adversely impacts post-natal survival,[4, 5] growth rate,[6] muscle and organ development,[7] carcass and meat quality,[8, 9] blood pressure,[10] and the stress (cortisol) response.[11, 12] Moreover, prenatal maternal stress in late gestation lowers post-natal immune function, reduces passive immunity and increases post-natal mortality.[13]

Hormones play a central role in fetal development by influencing tissue accretion, differentiation and maturation[14] and by altering both metabolism and gene expression in fetal

tissues. In normal pregnancies, cortisol, thyroxin, insulin and insulin-like growth factor (IGF) help to ensure that fetal growth is commensurate with nutrient supply.[15] Reduced thyroxin and elevated cortisol in particular, interrupt the accretion and differentiation of a wide range of tissues including the lungs, liver, kidney, muscle, fat and gut, and result in an asymmetrical type of fetal growth retardation, characterized by a long-thin body confirmation and low body mass index (BMI) at birth.[12, 16] Cortisol, which is produced in the adrenal gland in response to stress, is regulated through a number of complex neural and hormonal feedback pathways along the hypothalamic-pituitary-adrenal (HPA) axis. It is clear that while a physiologic rise in fetal cortisol in late gestation (after day 95) is crucial for the fetus' preparation for extra-uterine life,[15] excess cortisol at any other stage of gestation is detrimental to the pig's health and survival.[17]

The primary objective of this experiment was to investigate the effects of prenatal maternal stress occurring in early- and mid-gestation on fetal development, post-natal health and performance. The secondary objective of this experiment was to investigate the impact of birth weight on post-natal health and immunologic function. In this experiment, maternal stress was artificially induced using dexamethasone (Dex) administered by daily intramuscular injection. Like cortisol, dexamethasone has anti-inflammatory and immunosuppressive effects, but its activity is long acting and it is twenty times more potent than cortisol (Table 1). These gestational stages were selected as they are particularly critical to the development of numerous vital organ systems including those potentially impacting the immune and stress responses. The endpoints investigated in this experiment included litter size, piglet developmental characteristics (birth weight, body conformation, organ weights), piglet performance (growth rate, mortality and morbidity risk), immunologic function (passive immunity, antibody and cellular immune response, inflammatory cytokine response, leukocyte subset populations), haemogram and the

stress response. This experiment has relevance for the Saskatchewan swine industry in the areas of disease control, public health, animal welfare and efficient pork production.

MATERIALS AND METHODS:

Farm and animals: Seventy-seven mixed parity PIC pregnant females, located at the Prairie Swine Centre Inc., Floral, SK. and equally divided into 3 treatment groups were used in this experiment. To facilitate tissue collection and immunological testing, between 4 and 7 pregnant females, blocked by treatment group were added to the study weekly from July to November 2006. Pregnant gilts (parity 0) were excluded as they were located in a separate parity-segregated gestation-farrowing barn. The pregnant females included in the experiment were in good body condition, and were housed in the same gestation room in individual single gestation stalls. To decrease age variability of the studied offspring, sows were only included if they received their first AI service with PIC-Line 337 semen on Sunday, Monday or Tuesday, and exhibited a wean to first service interval of less than 7 days. Prior to weaning, all females were vaccinated for parvovirus, leptospirosis and erysipelas (Farrowsure B, Pfizer Animal Health, Kirkland, QC). Artificial insemination was conducted as per standard procedures on the farm, and all females was confirmed pregnant by RTU by gestation day (GD) 25 (Agroscan A7, SEC Repro, Ange Gardien de Rouville, QC). Sows were fed a commercial gestation diet once daily in the early morning according to stage of gestation as follows: 2.2-2.5 kg/d for the first 3 weeks following service; 2.75-3.25 kg/d adjusted as required based body condition until GD 90; then increased 1.0 kg/day until GD 112. All other aspects of animal care and husbandry were typical of commercial swine farms, and all procedures were conducted in accordance with the Canadian

Council for Animal Care, under the University of Saskatchewan's Assurance of Animal Care Protocol # 20040101.

Group allocation & dexamethasone administration: On approximately GD 30 (each Tuesday), eligible sows were weighed, and randomly assigned to one of two dexamethasone groups, or served as controls. Sows in each of the dexamethasone groups received 0.2 mg/kg (1 mL/25kg) dexamethasone (Dexamethasone 5, Vetoquinol, Lavaltrie, QC) IM in the neck for 7 consecutive days either from GD 30 to GD36 inclusive (Dex-30; n=27) or from GD60 to GD66 inclusive (Dex-60; n=27). All dex injections were given at 9 am, about 1 hour post feeding. The control sows (CRTL; n=25) were managed similarly in all other aspects, but received no injections. A placebo was not used due to the potential stress response that may have been induced through its daily parenteral administration.

Salivary dexamethasone measurements: A saliva sample was collected from all dex-treated sows immediately prior to administration (T0) and 5 hours post administration (T5) on GD 30-36 (for the Dex-30 group) or on GD 60-66 (for the DEX-60 group). A saliva sample was collected from each control sow on at T0 and T5 on GD 30-36 and GD 60-66. Saliva was also collected from all sows on GD 50 and GD 90. Saliva was collected using a cotton salivette (product #51.1534, Sarstedt, St. Leonard, QC) tied with dental floss to flexible plastic rod. After sampling, the salivettes were immediately refrigerated, and at the end of the day centrifuge for 20 minutes at 3,000 rpm, and the saliva frozen at -20°C. A subset of saliva samples (3 per sow; GD 30-T0, GD 34-T0, GD 34-T5 from the DEX-30 and CTRL sows, and GD 60-T0, GD 64-T0 and GD 64-T5 from the DEX-60 sows) were assayed for dexamethasone using a commercial qualitative ELISA kit purchased from Neogen Corp. (Lexington, KY). The dexamethasone assay was performed at the Lacombe Research Station, Lacombe, Alberta as per the manufacturer's instructions, but a standard curve was developed to provide for quantitative analyses. Standard concentrations of

dexamethasone (Sigma-Aldrich) were constructed in assay buffer (PBS) and ranged from 10 – 1000pg/ml. Assay sensitivity was calculated from multiple standard curves (n=12) on a single 96-well plate. Sensitivity was defined as the smallest signal detectable from zero and was taken to be the concentration equivalent to the mean absorbency count for the zero standard minus 1 standard deviation. Thus, the assay sensitivity for dexamethasone was 0.56 pg/ml (1.43 pmol/L). Dexamethasone concentrations in samples were interpolated from a 4-parameter fit model of standard concentration vs. absorbance. Positive and negative control samples were included with the kit. The positive control serum was diluted 1:10 with assay buffer so that the concentration could be read directly from the standard curve. The inter assay c.v. for the positive control was 4.9% at a mean concentration of 259pmol/L.

Farrowing procedures: Sows were moved from their gestation stall into the appropriate farrowing room on approximately GD 110. Sows that had not farrowed naturally by GD 114 were induced with a prostaglandin analogue cloprostenol (Estrumate, Schering Plough Animal Health, Pointe Claire, QC), to farrow the following day. Two 0.75 mL (375 ug) injections were administered in the abdominal oblique muscle in an extra-label manner as previously described[18] at approximately 8:30am and 2:30pm. Sows that did not farrow over night and showed no signs of milk let-down were re-induced in the same manner the morning of GD 115. Farrowings were monitored and sows assisted if complications arose. Farrowing assistance including the use of oxytocin, and was carried out by farrowing technicians as per standard barn operating procedures. The day of farrowing was considered day 0 (D0) of the experiment.

Colostrum immunoglobulin measurements: A 1-2 mL sample of colostrum was collected from each sow from a minimum of 3 teats on the day of farrowing. Sows farrowing overnight were sampled the following morning at the earliest opportunity. Colostrum was frozen at -20°C

pending analysis at the Prairie Diagnositic Services, Saskatoon, Saskatchewan for porcine immunoglobulin (IgG) as previously described[19].

Litter characteristics, birth weight and body conformation: On the day of birth (D0), and prior to any cross fostering, the number of live born, stillborn and mummified piglets in each litter was recorded. The crown rump length (CRL), girth diameter (measured immediately behind the front legs), and birth weight (BW) of each live and stillborn piglet were also obtained. The body mass index (BMI; birth weight divided by crown rump length squared), and BW:CRL, BW:girth and girth:CRL ratios were calculated for each piglet. Also on D0, pig processing was completed included the clipping of teeth, ear notching for identification purposes and the IM administration of 1mL (200 mg) iron dextran (Ferroforte, Vetoquinol, Lavaltrie, QC). When farrowing started too late in the day and was not completed by the end of the work day, the processing and weights were completed the following morning. On approximately D5 tails were docked and male piglets were castrated.

Organ weights: On D0 one piglet per litter of average birth weight was euthanized using a CO_2 chamber (100% CO_2 piped into a 14"x16"x 20" Rubbermaid container at ~1 L/min for approximately 10-12 minutes). Immediately after death, each piglet was weighed, and then dissected to attain the weight of the brain, liver, spleen, thymus, lung, heart, kidney and adrenal glands. On D13 (range 12-14) one additional piglet of average birth weight from each biologic litter was euthanized and dissected in the same manner. The ratio of each organ relative to the brain, a traditional predictor of intrauterine growth retardation, was calculated and compared among treatment groups.

Cross-fostering procedures: Judicious cross-fostering of newborn piglets was encouraged to enhance piglet survival and growth. As much as possible, the piglets suckled their biologic dam for the first 24 hours and split suckling was used as required. As it was our aim was to provide

the best suckling environment for each piglet, cross-fostering was conducted between sows regardless of treatment group to even out piglet size and number, but piglets were not fostered outside of the farrowing room or farrowing week except in emergency situations. All cross fostering was completed within 48 hours of birth, and piglets were rarely moved from the litter thereafter. No milk replacer or creep feed was provided during the suckling period. These cross fostering procedures created the best opportunity for each piglet to express its genetic potential, and also indirectly blinded the farrowing staff to treatment group because each suckling litter was potentially of mixed origin, and cross-referencing an individual piglet to its biological dam was possible only by reading an ear notch.

Neonatal immunoglobulin levels: Twenty-four hours after birth, 2-3 mLs blood was collected from three full sibling piglets representing the piglets with the lowest, average and highest birth weight from each biological litter. The blood, collected from the jugular vein into a plain glass vial while the pig was restrained in dorsal recumbency, yielded about 1 mL serum after centrifugation at 1,800 RPM for 10 minutes. Sera was frozen at -20°C pending total porcine IgG analysis using radioimmunodiffusion assay[19] (RID) at the Prairie Diagnostic Services, Saskatoon, SK..

Lipopolysaccharide (LPS) induced inflammatory cytokine production of whole blood: At approximately 12 days of age (range 11-14), 5 mLs blood was collected from three full sibling piglets representative of the lowest, average and highest birth weight from each biological litter. The blood was collected from the jugular vein into heparinized vials while the pig was restrained in dorsal recumbency. Within 2-3 hours of collection, one mL aliquots of heparinized whole blood were placed in 48 well TC plates in duplicate. One well was stimulated with 1 ug/mL LPS and the other served as an unstimulated saline control. Plasma was harvested at 24 h post-stimulation and frozen at -80°C pending cytokine quantification. The plasma was thawed,

diluted 1:5 (IL-1 & IL-8) or 1:3 (TNF-alpha) in ELISA diluent and the levels of IL-1, IL-8 and TNF-alpha measured by ELISA (R&D Systems Duoset Kits, Minneapolis, MN) as per manufacturer's protocol, with the following modifications. *Day 1:* The 96 well plates were coated with capture antibody in carbonate coating buffer (1:180 dilution) and left to incubate at 4°C overnight. PBST washing solution was prepared (0.01M PBS + 0.05% Tween 20) and left at room temperature. *Day 2:* Each deep well containing plasma was thawed in a 37°C water bath. Each plate was washed 4x by submersing in PBST. The plates were then blocked for one hour at 37°C with 100 uL of blocking solution (1% BSA in PBS) in each well. While blocking, the standard dilutions (defined by each kit) were made in Eppendorf tubes using the reagent diluent (working buffer + 1% BSA). The plates were washed 4x in fresh PBST. Plasma samples were diluted in the ELISA plate by adding 80 uL ELISA buffer (0.01M phosphate buffer, pH 7.2, with 0.75 M NaCl, 0.05% Tween 20) containing 1% BSA to all of the sample wells. Then 20 uL of plasma from the deep well was transferred to the ELISA plate in duplicate, leaving the last two wells of each row for the standard dilutions which were also added at this time to a total volume of 100 uL per well. The plates were then covered and incubated overnight at 4°C. *Day 3:* Plates were washed 4x in PBST. 100 uL of the detection antibody (dilution was kit dependant and diluted in reagent diluent) was added to each well and incubated for 2 hours at room temperature. Plates were washed 4x in PBST. 100 uL of the Strep-HRP was added to the wells in a 1:200 dilution in reagent diluent and incubated for 20 minutes at room temperature. The plates were covered to keep them out of the direct light. The plates were washed 4x in PBST. 100 uL of TMB was added to each well for 10-20 minutes at room temperature and the plates were covered to keep them out of direct light. 50uL of Sulfuric Acid was added to each well to stop the reaction. Each plate was then read using a plate reader at 450nm with 550nm for correction. The

stimulation response was determined by subtracting the OD (saline control) from the OD (LPS-stimulated).

Leukocyte subsets: From the same 12 day old piglets, approximately 4 mLs of blood was collected and anticoagulated with EDTA. From each, a 1 mL aliquot was submitted for complete blood count (CBC) and white blood cell differential count (DIFF) at the Prairie Diagnostic Services, Saskatoon, SK.. The remaining blood was prepared for flow cytometry (PDS Diagnostic Immunology) as follows. Five tubes per blood sample, were prepared, each containing a pair of monoclonal antibodies or isotype controls. One microgram of each monoclonal antibody or isotype control directly conjugated to either FITC, PE or Alexa Flour 488 (Table 2) was added to 200 uLs of whole blood, and incubated for 30 minutes at 4°C. The RBC's were lysed with 2 mL FACSLyse (BD Bioscience, San Jose, CA) per tube and held at room temperature for 6 mins. The cells were then re-suspended in 500 uL of 0.5% neutral buffered formalin (0.2% formaldehyde v/v) and were refrigerated overnight. The following morning, a minimum 30,000 cells were counted using a Coulter Epics Elite flow cytometer, at approximately 500-700 cells per second (Saskatoon Cancer Centre, Saskatoon, SK.). Analysis of the leukocyte subsets was performed using Expo-32 software (Applied Cytometry Systems, Dinnington, Sheffield, UK) as follows. Firstly, the population of leukocytes excluding the cellular debris with very low forward and scatter was gated, and this population was used for all subsequent analysis. Gates for each leukocyte phenotypic marker were established to best capture the cell population under examination (Figures 1-3). For consistency, the gating specifications were near identical across all samples but minor adjustments were necessary for some individual samples to coincide with slightly stronger or weaker staining intensity. In such samples, the position of a gate was shifted left/right or up/down as appropriate, but the overall size of the gate and the area it captured remained unchanged (Table 3). The proportion of cells

within each gate was recorded, and the absolute number of each leukocyte subtype was calculated by multiplying the absolute WBC count number (derived from the CBC) by the proportion of cells captured within each gate.

Suckling piglet performance and health: The body weight of all surviving piglets was obtained at 20 days of age, just prior to weaning, and the average daily gain (ADG) from birth to day 20 was calculated. Three litters were weighed on D19 due to a late farrowing date and the ADG calculation was adjusted accordingly. During the suckling period, all piglet morbidity (treatments) and mortality was recorded including reason and date. Litters were weaned at 19-22 days of age and transferred to the nursery.

Nursery performance and health: At weaning pigs were sorted by body weight (small, medium, large), treatment group and gender into groups of 10-13 pigs depending on the production week into 2.5m x 1.04m pen providing 0.2 to 0.26 m^2 per pig. Within each week, pen density was consistent among all pens. Before weaning, each litter was identified and uniquely spray marked to ensure that piglets from each treatment group and litter were mixed as evenly as possible into the nursery pens. The pigs were individually re-weighed on days 15 and 30 post-weaning, and the growth rate for each period was calculated. The identity of all pigs that died or were treated during the nursery period was recorded, along with the date and reason.

Cortisol (stress) response naturally induced by weaning: One male and one female piglet from each biological litter and of average body weight at time of weaning were selected to measure the cortisol (stress) response to weaning and regrouping. On the day prior to weaning, and on hours 1, 3, 5 and 23 post-weaning, 0.5 to 1 mL blood was collected from the jugular vein of each selected pig while restrained in dorsal recumbency. As restraint by itself may have induced a cortisol (stress) response, care was taken to ensure the procedure was completed as quickly as possible, and sampling requiring more than 60 seconds to complete was accounted for in the

statistical analysis. After clotting, the serum was separated and frozen at -20°C pending cortisol quantification by an in-house ELISA at the Lacombe Research Station. Rabbit anti-cortisol serum (Fitzgerald, MA) raised against a cortisol-3-BSA conjugate, was diluted with carbonate/bicarbonate buffer (pH = 10) to a final dilution of 1:40,000 and coated on the surface of 96-well mictrotitre plates. A cortisol-3-HRP conjugate (Y J Bioproducts, CA) was used as the tracer and tetramethylbenzidine (TMB) as the enzyme substrate was purchased from MJS Biolynx Inc. (ON). Hydrocortisone, purchased from Sigma-Aldrich (ON), was initially dissolved in ethanol to provide a stock solution of 10mg/ml and subsequently diluted in assay buffer to give a standard curve range of 10 – 1000pg/50µl. Sera (50µl) were assayed in duplicate and all samples giving a c.v. >15% were repeated. The intra-assay coefficient of variation at a mean concentration of 40nmol/L was 6.0% and the inter-assay c.v. at a mean concentration of 32.2 nmol/L was 11.8%. Assay sensitivity was 5pg/50µl and the relative cross-reactivity with dexamethasone <0.001% at 70% displacement of labeled antigen. The cortisol response on hours 1, 3, 5 and 22 post-weaning was calculated as a percentage over the baseline (D-1) level.

Humoral (antibody) immune response: Four piglets in each biological litter were vaccinated intramuscularly with 2 mL of a commercial *Actinobacillus pleuropneumoniae* (APP) vaccine (PneuPac, Schering Plough Animal Health, Pointe Claire, QC) one day after weaning and 14 days later. The vaccinated pigs equally represented the heaviest and lightest piglets based on their weaning weight. On day 30 post weaning (15 days post second APP vaccination), sufficient blood was collected from the jugular vein from each vaccinated pig to yield 2 mL serum after centrifugation. The level of APP serum antibody specific for serotypes 5 and 7 was measured in each sample by ELISA at the University of Montreal, and was used to estimate the antibody immune response among treatment group.

Phytohaemagglutinin (PHA) induced delayed type hypersensitivity (DTH) skin test: The piglets selected to evaluate the antibody immune response were also used to assess cellular immunity. One day after weaning, two intradermal injections were made using new 28G x 0.5" needles and a 1 mL insulin syringes approximately 1-2 cm in from the margin of the ear on the dorsal surface. In the left and right ears respectively, 0.1 mL (250 micrograms) phytohaemagglutinin (PHA) or 0.1 mL saline were administered. Prior to administration the thickness of the ear was measured at the administration site using a constant tension micrometer. Twenty-four hours post-injection, the size and thickness of the resulting lesions were measured with a caliper and constant tension micrometer respectively. The change in size and thickness was calculated.

Statistical analysis: The outcome data for each treatment group was examined descriptively at the level of the sow or for piglets within litters as appropriate. Generalized linear models were used to estimate the difference in outcome measures across treatment groups (SAS for Windows version 8.2; SAS Institute Inc, Cary, North Carolina). Outcomes examined using a binomial distribution and logit link function included proportion of stillborn piglets; the risk of piglet morbidity and mortality. Count variables were examined using a Poisson distribution and included total born and total number of mummies per litter, and total live born and total stillbirths per litter (as outcomes potentially related to treatment). All continuous outcomes examined using a normal distribution. Estimates of differences in binomial and count outcomes measured for each piglet were adjusted for clustering within litter using generalized estimating equations (PROC GENMOD) and estimates for continuous piglet outcomes were adjusted for clustering within litter by including a random intercept for litter in the model (PROC MIXED). Other biological or management factors considered potential confounding variables in the analysis included gender, sow breed and parity, birth weight, farrowing week, biological litter size, weaning age, farrowing induction, gestation length, surrogate sow parity, surrogate sow ID,

surrogate day 2 and day 19 litter sizes. Potentially important biological or management risk factors were retained in the final model if they changed the regression coefficient for the treatment variable by more than 10% or if they were statistically significant ($P < .05$). All differences between treatment groups were considered statistically significant where $P < 0.05$, and trending towards significance at $0.05 > P < 0.1$. The impact of piglet birth weight was evaluated in two ways. For the immunological and haematological (CBC & RBC levels) outcomes, a subset of piglets from each litter was selected representing high (HBW), average (ABW) and low birth weight (LBW) categories. The impact of the birth weight was estimated in terms of weight category (high, average, low). For other outcomes including growth performance and morbidity risk, birth weight was included in the statistical model as a continuous variable, and a regression coefficient calculated.

RESULTS & DISCUSSION

Impact of pre-natal dexamethasone administration:

Dexamethasone was evident in the saliva of nearly all animals regardless of treatment group. Moreover, saliva samples collected prior to the first dexamethasone administration on GD 30 (time 0) were positive (Table 4). These results were a complete surprise and initiated an investigation for possible causes. While salivary dexamethasone levels were numerically higher in Dex-30 versus CTRL sows at D34-T0 & D34-T5, there was considerable between-animal variation across in groups. Our investigation into the source of the dexamethasone continues but at the time of writing we have yet to determine why these dexamethasone levels were unusually high in the untreated animals. At present, we have not ascertained if these findings are the result of an assay cross-reaction, or due to dexamethasone contamination of the experimental sows or

the diets the sows were fed. While treatment group differences are reported below, at the time of writing we are uncertain to their biological significance.

There were no dex-treatment group differences in litter size, although total born and live born per litter were numerically lower in the Dex-30 group (Table 5). Detecting litter size differences were not our primary objective, thus the experiment had limited power with this respect. The litter size differences may have been statistically significant if a larger sample size had been used, and to this end may warrant further investigation.

The BW:CRL ratio and BMI were significantly lower in the Dex-30 group compared to CTRL ($P<0.05$ for each), and trended lower in the Dex-60 group compared to CTRL ($P=0.063$ for BW:CRL; $P=0.092$ for BMI). However there were no statistical differences between the Dex-30 and Dex-60 groups for either trait. These results have a biological basis and demonstrate that pre-natal dexamethasone administration potentially altered fetal growth resulting in a long-thin body conformation at birth.

The adrenal:brain ratio was significantly lower in the Dex-60 compared to the Dex-30 ($P<0.02$) group, and trended lower compared to CTRL pigs ($P=0.06$). This finding suggests the presence of a functional negative feed back mechanism after GD 60 imposed by dexamethasone on adrenal gland development. Based on our data, this feedback mechanism appears not to be functional at GD 30. There were no group differences in baseline cortisol level, or the cortisol response at 1, 3, 5, or 23 hours following natural weaning. However, the use of a more robust *in-vivo* test such as the adrenocorticotropic hormone (ACTH) induced cortisol response would be warranted in future research to clarify the biological significance of the lower adrenal:brain ratio in the Dex-60 group.

The development of the heart, lung, liver spleen or thymus was not impacted by dex administration. However, the kidney:brain ratio was significantly higher ($P<0.03$) in the Dex-30

pigs compared to Dex-60 or CTRL pigs. This finding may be spurious and is of uncertain biologic significance.

The Dex-60 group expressed faster growth rates during the nursery phase of the experiment. During days 20-50, the Dex-60 grew 26.4 g/d faster than CRTL pigs (P=0.01). During days 34-50 the Dex-60 grew 23.8 g/d faster than CRTL pigs (P=0.056) and 36.0 g/d faster than Dex-30 pigs (P<0.05). As a result of faster growth during the later nursery stage, overall ADG from birth to day 50 (ADG 0-50) was significantly higher in the Dex-60 group than in the CRTL (17.4 g/d, P<0.02) and the Dex-30 (17.7 g/d, P<0.02) groups. There were no treatment group differences in average daily gain (ADG) during the suckling (days 0-20) or early post weaning (days 20-34) periods, nor were there group differences in mortality or morbidity. While the higher growth rates in Dex-60 pigs may seem contrary to expectation, it is not entirely unexpected and may suggest compensatory growth. When comparing post natal growth performance in low versus high birth weight piglets, Poore et al.[6] found higher fractional growth rate (kg gained per day per starting body weight) in low compared to high birth weight pigs during the first month of life, and between 3 and 12 months of age. While in our experiment, the pigs in each treatment group were of similar body weight, prenatal dexamethasone administration may have altered fetal growth and development in a similar manner to that which low birth weight piglets experience. Thus, it is possible that the Dex-60 group experienced a significance *in-utero* insult during a critical period of muscle development. In the fetal pig, secondary muscle fibres begin to develop beginning GD 55 and some *in-utero* insults such as uterine overcrowding that occur during this period negatively impact secondary muscle fibre development,[7] ultimately reducing the total number of muscle fibres at birth and throughout post-natal life. As part of a compensatory mechanism in affected pigs, individual muscle fibres grow faster post-natally, and become larger in diameter compared to the muscle fibres of normal pigs born. Since very large diameter muscle

fibres (aka giant fibres) are associated with poor meat quality including high drip loss, low pH_{45}, [20] the impact of pre-natal maternal stress on muscle fibre number and carcass quality should be further investigated.

The antibody (IgG) concentration in the sow's colostrum was not impacted by treatment group and pre-natal dexamethasone administration had no impact in the antibody (IgG) concentration in the piglet's serum at 24 hours of age.

With regards to the other immunologic assays used in this experiment, pre-natal dexamethasone administration had no impact on the LPS-induced cytokine production, cellular immunity (PHA-induced delayed DTH skin test), or antibody production in response to APP vaccination. However at 13 days of age, the absolute number of $CD8^+$ lymphocytes were significantly lower in the Dex-60 ($P = 0.01$) and trended lower in the Dex 30 pigs ($P = 0.07$) compared to CRTL suggesting prenatal stress negatively impacted the development or maturation of this subpopulation of cytotoxic T cells. In addition, the absolute number of granulocytes was lower in the Dex-30 compared to the Dex-60 pigs (P=0.03). After controlling for birth weight, no group differences with respect to any parameters measured in the complete blood count (CBC) were evident.

Impact of birth weight:

Birth weight positively impacted growth rates from birth to 50 days of age, and heavier pigs grew faster throughout the suckling and nursery periods. For every additional 100 grams in birth weight, ADG during lactation (days 0-20) increased by 9.8 g/d (P<0.0001). For every additional 100 grams in birth weight, ADG during the nursery period (days 20-50) increased by 9.0 g/d (P<0.0001). For every 100 gram increase in birth weight, the risk of morbidity for any reason dropped by 0.08% ($P = 0.02$).

LBW piglets had significantly lower passive immunity levels than HBW ($P<0.0001$) and ABW ($P = 0.003$) piglets. Moreover, the light pigs at weaning demonstrated an inferior reaction to the PHA-induced (DTH) skin test compared to heavy pigs at weaning ($P = 0.009$). These findings suggest that light or small piglets are at a clear disadvantage with respect to both passive and active cellular immunity from birth to shortly after weaning. By contrast, body weight at weaning had no impact on the antibody response following boosted APP vaccine.

At 13 days of age, LBW compared to HBW piglets had significantly lower absolute numbers of $CD3^+$ T lymphocytes ($P<0.0001$), $CD21^+$ B-lymphocytes ($P = 0.004$), $CD4^+$ T-helper lymphocytes ($P = 0.02$), $CD8^+$ cytotoxic T-lymphocytes ($P<0.002$), and gamma-delta lymphocytes ($P<0.0001$). Compared to HBW piglets, ABW piglets had significantly lower absolute numbers of $CD3^+$ T lymphocytes ($P = 0.02$), $CD8^+$ cytotoxic T-lymphocytes ($P = 0.007$), and gamma-delta lymphocytes ($P = 0.04$). Ironically, birth weight had no impact on total white blood cell (WBC) numbers. While changes in absolute lymphocyte numbers do not necessarily reflect immune dysfunction, these findings provide further support for the concept that the active immune response is compromised in pigs of below average birth weight.

Although not clinically anemic, LBW and ABW piglets had lower red blood cell (RBC) counts compared to HBW piglets ($P < 0.0001$ for LBW, $P = 0.02$ for ABW). Moreover, the red blood cells of LBW and ABW piglets compared to HBW piglets were larger and more variable in size ($P < 0.01$ for all measures). These findings are indicative of active production of red blood cell and the premature release of immature red blood cells into the general circulation.

CONCLUSIONS & RECOMMENDATIONS:

The primary objective of this study was to investigate the impact of prenatal maternal stress in early- and mid-pregnancy, on post-natal immune function, performance and health of the offspring using a dexamethasone administration model. While the dosage of dexamethasone used in the experiment was considered high by human experimental standards, appropriate therapeutic levels for pigs are not well understood. One potential limitation of this experiment was that dexamethasone administration rather than natural stress was used. Secondly, the dosage of dexamethasone used mimicked a very high level of stress that was potentially far in excess of that occurring naturally in normal production settings. While these are valid concerns, a natural stress model is very difficult to reproduce in commercial production settings while maintaining an appropriate level of animal care and welfare. More importantly, because this continues to be an emerging area of research, it was important to first investigate the potential immunologic impacts at high stress levels, and then proceed with subsequent evaluations in naturally occurring stressful situations if appropriate.

However, we were surprised and disappointed that dexamethasone was found in the saliva of all sows across all treatment groups. Furthermore, we still have an inadequate understanding why dexamethasone was measured in sows which had no prior history of dexamethasone treatment. It is theoretically possible that an unknown source of dexamethasone or a metabolite was unknowingly made available to the experimental sows. It is also possible that this finding may have been the result of human error related to improper dexamethasone administration or an inadvertent mix up in the sample identification after collection, however we can find no evidence to support any of these scenarios. In addition, there was no evidence of routine dexamethasone use on the farm other than that used in this experiment. It is our present understanding that the dexamethasone ELISA used was very specific and free of cross-reactions. Moreover, saliva

samples collected from untreated animals in another farm and tested simultaneously were clearly negative. More importantly, these findings highlight an urgent need to continue investigating this issue, yet they admittedly put some of the experiment's primary outcomes at risk of being insignificant. We have chosen to report them nonetheless. These findings also have potentially severe implications for the swine industry, because we have found the evidence of a drug and are unable to track its source. Our investigation will continue but we are relieved that a small number of sows and feed samples collected at the experimental farm in late 2006, about 1 year after the implementation of this experiment, were free of dexamethasone.

Under the conditions of this study, prenatal dexamethasone administration adversely impacted fetal development in several ways. Firstly, the dex-administration resulted in the birth of long-thin piglets, particularly when administered in early gestation. This finding has a biologic relevance, in that long-thin conformation at birth are detrimental to post-natal health. Human babes with long-thin conformation at birth are at higher risk of hypertension, glucose intolerance and cardiovascular diseases later in adult life.[1-3] In pigs, long-thin body confirmation at birth adversely impacts post-natal stress response, basal cardiovascular function and glucose metabolism.[10, 12, 21]

Pigs in the Dex-60 group had faster growth rates which is suggestive of compensatory growth and altered *in-utero* secondary muscle fibre development. Moreover, when the fibre number is low at birth, individual muscle fibres grow more quickly during post natal development.[22] Low fibre number increases the risk of structurally abnormal "giant" fibres which are associated with poor meat quality, including higher drip loss and brightness, and lower $pH_{45\ min}$.[20] Therefore, the potential impact of pre-natal maternal stress in the late gestation warrants further research because of its potential impact on growth performance, carcass and quality.

We found little evidence that prenatal dexamethasone treatment adversely impacted post-natal immune function or stress response. Our immune function measures included *in-vivo* and *in-vitro* assays reflecting cellular and antibody immunity. A potential limitation of this experiment was that the immunologic assays used were not sensitive enough to detect small differences in immune development, nor were they linked to a clinical entity. Since the immune system is designed to fight infection, this experiment would have been strengthened if a specific disease such as porcine circovirus was used as out outcome. Nonetheless, prenatal dexamethasone treatment adversely impacted the number of $CD8^+$ lymphocytes, and potentially the number of granulocytes in the Dex-30 group. Both cell types are critical for the immune response: $CD8^+$ lymphocytes are involved in MHC Class I restricted cytotoxic killing of intracellular pathogens, and granulocytes in the defense against bacterial infections. Additional research would be useful to validate these results, specifically to determine if these changes result in any functional change in the immune response against a specific disease.

The secondary objective of this study was to investigate the impact of birth weight on post-natal health and immunologic function. We are pleased with these results because they are profound and have biologic basis. As previously stated, low birth weight in pigs negatively impacts many traits or characteristics essential for efficient swine production including survival, growth rate, and carcass and meat quality. This research further supports the concept that small birth weight pigs are not only small, but they are developmentally altered which has negative implications for many organ systems including the immune system. Our results imply that small birth weight pigs are at greater risk of disease in early life due to inferior passive and active immunity. Moreover, some aspects of the immune response were inferior in average birth weight pigs, which highlights that high birth weight pigs are at a considerable advantage not only nutritionally, but also immunologically. Unfortunately, in the modern swine industry, too little

attention is focused on enhancing birth weight through management or genetic strategies. As birth weight is negatively correlated with litter size, genetic advancement in litter size and the selection hyper-prolific litters and genotypes may in fact cause a reduction in birth weight in some operations or genetic lines. Thus, this research is of paramount importance to the swine industry, in that fully understanding the *value* of birth weight is critical to disease resistance and health status. Enhancing birth weight is essential to efficient pork production and the sustainability of the western Canadian swine industry, and warrants ongoing research support.

IMPLICATIONS FOR THE SWINE INSTRY

- Under the conditions of this experiment, maternal dexamethasone administration between gestation day 30-36 and gestation day 60-66 had limited impact on immunologic function. These findings suggest that prenatal maternal stress has minimal functional impact on most aspects of the developing fetal immune system.
- However, maternal dexamethasone administration between gestation day 60-66 negatively impacted the number of $CD8^+$ cytotoxic T lymphocytes. This is biologically relevant because this subset of immune cells is critical to fight intracellular pathogens such as viruses.
- Maternal dexamethasone administration between gestation day 30-36 negatively impacted fetal growth resulting in the birth of long-thin piglets with low BW:CRL ratio and BMI. This is biologically relevant because long-thin piglets are at higher risk of altered post-natal health.
- Maternal dexamethasone administration between gestation day 60-66 reduced the mass of the fetal adrenal gland, suggesting the presence of a negative feedback of dexamethasone on adrenal gland growth. While we did not find any evidence of an altered stress response, the

use of a more robust test in future research is recommended to further understand the biological relevance of this finding.

- Maternal dexamethasone administration between gestation day 60-66 resulted in faster growth rates during the nursery period between 20 and 50 days of age. This finding, while appearing counter-intuitive, may have a biologic basis and suggests that muscle cell development may have been adversely impacted at a critical period of fetal development when secondary muscle fibres are developing. This may have potential implications for the pork producers because low secondary muscle fibre number is associated with poor meat quality in some breeds.
- Light and average birth weigh pigs demonstrated slower growth rates, higher risk of morbidity, and lower passive immunity levels than heavy birth weight pigs.
- Light, and to a lesser extent average birth weigh pigs, demonstrated a diminished cellular immune response to intradermal PHA administration, as well as lower T and B lymphocyte subsets, suggesting a potentially inferior active immune system compared to heavier pigs.
- While not clinically anemic, light and average BW piglets demonstrated lower red blood cell counts and the compensatory release of immature red blood cells into the general circulation.
- Enhancing birth weight is of considerable value for the swine industry and is critical for maintaining efficient growth and enhancing the health status of swine farms.

ACKNOWLEDGEMENTS

At the time of writing, the statistical analysis for the project has just been completed. The preparation of peer-reviewed papers and research abstracts is anticipated in the near future. Appropriate acknowledgements to the Department will be made in these publications, as well as in all oral research presentations made in the future. One oral presentation of this research is presently scheduled for November 2007, at the Western Canadian Association of Swine Veterinarian's annual meeting in Saskatoon.

Once again, I wish to that the Department for their generous contribution to this project. Our work in this area of research will continue and will build on the results of this experiment.

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Appendix I

TABLES & FIGURES

Table 1. The comparative properties of commercially available glucocorticoids in humans

Glucocorticoid drug	**Biological half-life**	**Equivalent anti-inflammatory dose (mg)**
Short- acting		
Cortisone	8 to 12 hours	25
Hydrocortisone		20
Intermediate-acting		
Methylprednisilone	18 to ours	4
Prednisilone		5
Prednisone		5
Triamcinolone		4
Long-acting		
Betamethasone	36 to 54	0.6
Dexamethasone		0.75

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Table 2. Monoclonal sources and fluorescent label conjugations used in the flow cytometric analysis

Tube	Phenotypic Marker	Monoclonal Source*	Product code	Fluorescent label	Detection of cell subpopulation
A	CD 3	Southern Biotech	4511-02	FITC	All T-lymphocytes
A	CD 21	"	4530-09	PE	B lymphocytes
B	CD 4	"	4515-02	FITC	T helper lymphocytes (MHC II)
B	CD 8	"	4520-09	PE	Cytotoxic T-lymphocytes (MHC I)
C	Monocyte Granulocyte	"	4525-09	PE	Monocytes, granulocytes (macrophages, neutrophils) in blood; less than 5% lymphocytes
C	Gamma-Delta (GD) TCR	VMRD	PG92A	Alexa Flour 488 (FITC-like)	Null cells expressing GD T cell receptor
D	Control	Southern Biotech	0103-09 0102-02	IgG2a (PE) IgG1 (FITC)	
E	Control	"	0104-02 0102-09	IgG2b (FITC) IgG1 (PE)	

* Southern Biotech – Birmingham, AL

* VMRD – Vet Medical Research Development - Pullman WA

Table 3. Positional adjustments made to individual samples to compensate for higher or lower staining intensity of phenotypic markers

Tube	Total no. samples	Marker (gate)	Number and description of positional adjustments required		
			None required	Adjustments compensating for lower staining intensity	Adjustments compensating for higher staining intensity
A	232	CD 3 (V)	225	6 – 0.02 to 0.05 log left	1 – 0.03 log right
		CD 21 (U)	232	none	none
B	232	CD 4 (V)	206	25 – 0.2 to 0.4 log left	1 – 0.2 log right
		CD 8 (Y)	232	none	none
		$CD4^+8^+$ (X)	229	3 – 0.3 to 0.4 log left	none
		$CD4^+8^+$ (Z)	232	none	none
C	226	GD (V)	198	23 – 0.05 to 0.1 log left	5 - 0.05 to 0.1 right
		Monocyte (Y)	226	none	none
		Granulocytes (Y)	198	28 – 0.05 to 0.4 log down	none

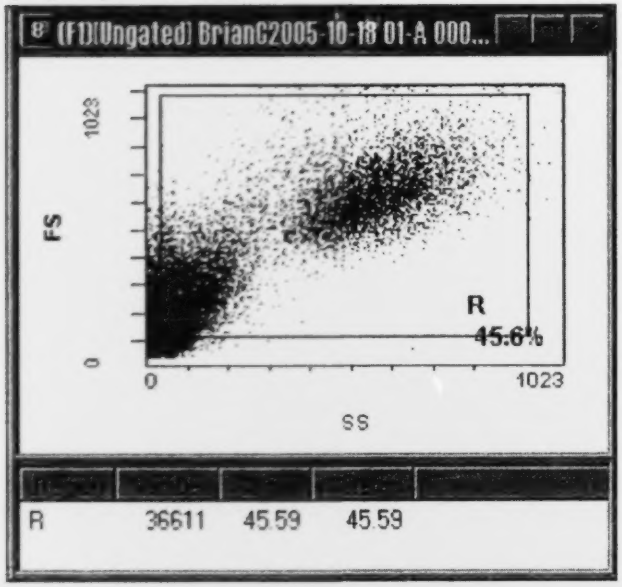
Table 4. Salivary dexamethasone levels pg/mL (SD) measured by ELISA by treatment group.

		Dex 30		Control		Dex 60	
Day of sample	**Time of sample**	**No. samples**	**Dex (pg/mL)**	**No. samples**	**Dex (pg/mL)**	**No. samples**	**Dex (pg/mL)**
30	T0	26	20.1 (24.0)	23	23.1 (24.1)		
34	T0	26	23.0 (32.6)	25	16.6 (28.3)		
	T5	25	30.1 (33.1)	24	24.4 (31.7)		
60	T0			Not completed		26	21.2 (23.5)
64	T0			Not completed		26	16.1 (23.0)
	T5			Not completed		26	20.2 (24.4)

Table 5. Simple descriptive statistics of litter size differences by dexamethasone treatment group.

Litter characteristics	**Dex 30 (n=27)**	**Dex 60 (n=27)**	**CTRL (n=25)**	**SEM**	***P* value**
Total born/litter	11.0	12.4	12.6	0.3	$P = 0.20$
Born alive/litter	10.0	11.3	11.6	0.4	$P = 0.32$
Stillborns/litter	0.9	0.9	0.9	0.1	$P = 0.98$
Mummified piglets/litter	0.1	0.26	0.20	0.06	$P = 0.43$

Figure 1. Typical leukocyte flow cytometric images following staining with CD3+ and CD21+ phenotypic stains. *Left:* Gate R contains cells with forward scatter (FS) and side scatter (SS) typical of the leukocyte populations of interest in all flow cytometric analysis. Excluded from gate R are large cells and cellular debris. *Right:* Image demonstrating typical gate placement and proportions of CD3+ (Gate V, pink) and CD21+ (Gate U, red) lymphocytes.



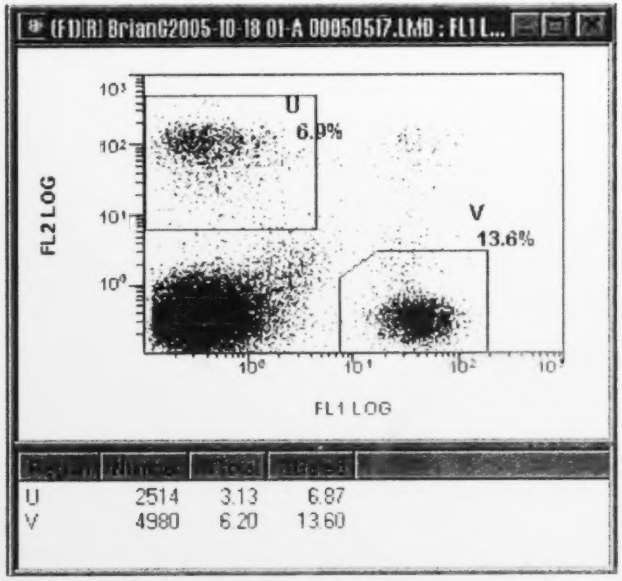
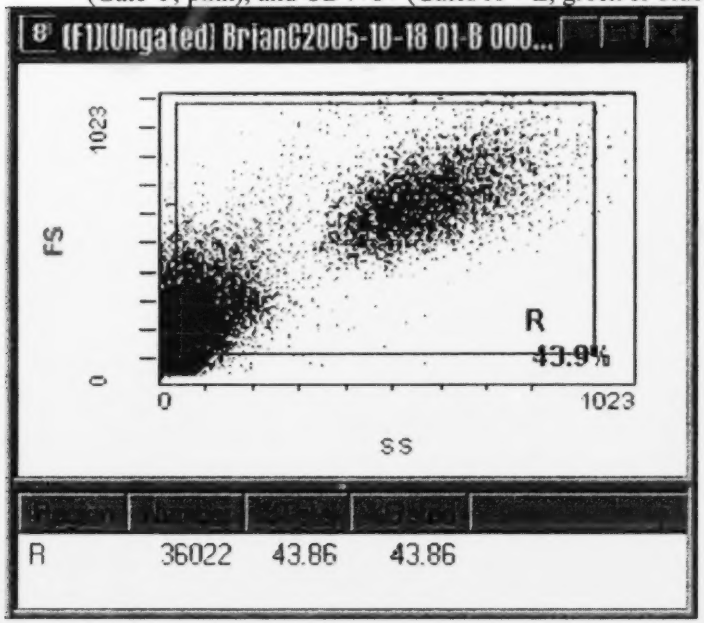


Figure 2. Typical leukocyte flow cytometric images following staining with CD4+ and CD8+ phenotypic stains. *Left:* Gate R contains cells with forward scatter (FS) and side scatter (SS) typical of the leukocyte populations of interest in all flow cytometric analysis. Excluded from gate R are large cells and cellular debris. CD4+ and CD8+ stained cells (blue, green and pink coloured) cells are noted within gait R. *Right:* Image demonstrating typical gate placement and proportions of CD4+ (Gate V, turquoise), CD8+ (Gate Y, pink), and CD4+8+ (Gates X + Z, green & blue) lymphocytes.



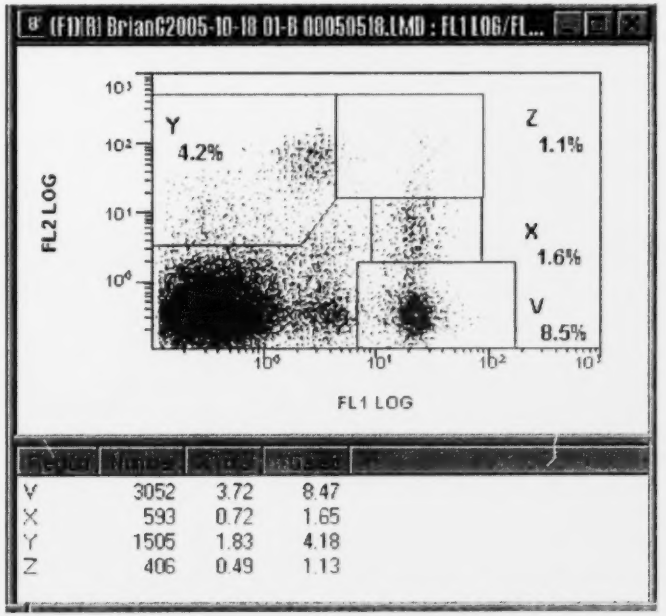
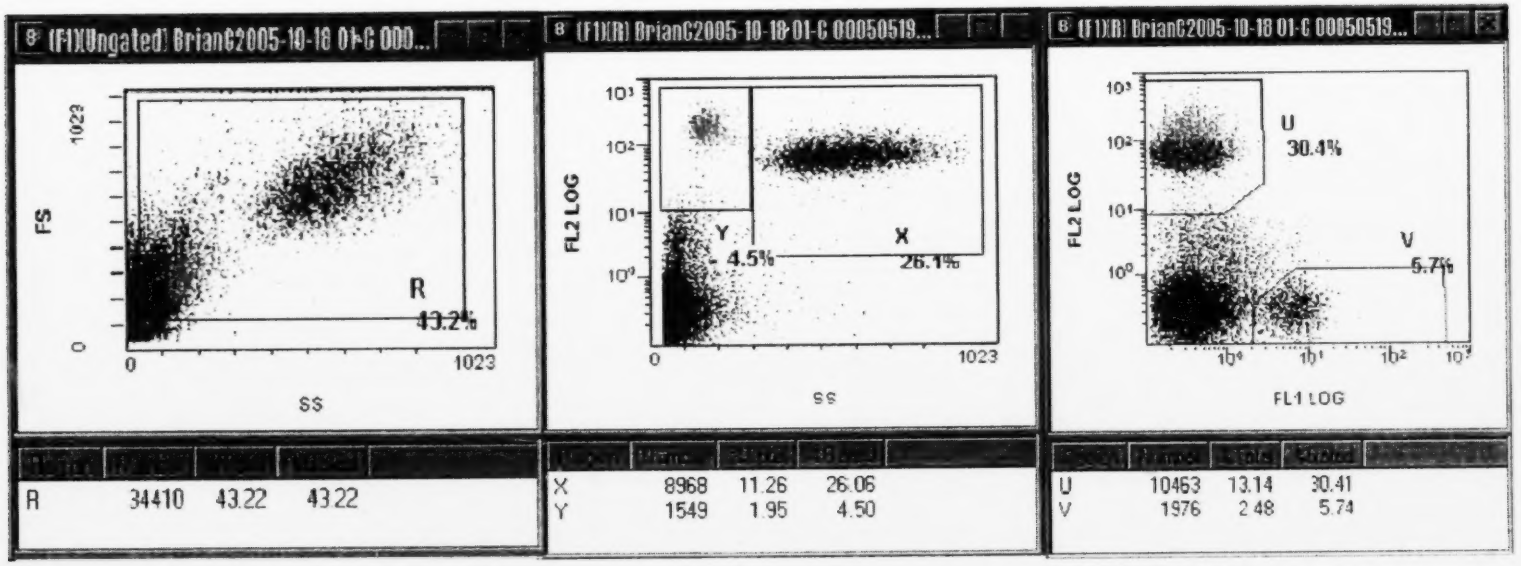


Figure 3. Typical leukocyte flow cytometric images following staining with the granulocyte and gamma-delta (GD) cell phenotypic stains. *Left:* Gate R contains cells with forward scatter (FS) and side scatter (SS) typical of the leukocyte populations of interest in all flow cytometric analysis. Excluded from gate R are large cells and cellular debris. GD cells, monocytes and granulocytes are stained blue, green, pink respectively within R. *Centre:* Image demonstrating typical gate placement and proportions of monocytes (Gate Y, green) and granulocytes (Gate X, pink). *Right:* Image demonstrating typical gate placement and proportions of GD lymphocytes (Gate V, blue).



OTHER DOCUMENTS

PERSONEL:

Ms. Crissie Auckland was hired as the project's research technician in May 2005, and continues to be employed in this and other research at the Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine. Ms. Auckland earned a certificate in Veterinary Technology from Kelsey College in 2002 and upon graduation worked at the Prairie Swine Centre in contract research and production for 3 years. As a result of this experiment her skill set as a research technician has expanded. She played a critical role in many laboratory assays in this other experiments.